A systematic analysis of the suitability of preimplantation genetic diagnosis for mitochondrial diseases in a heteroplasmic mitochondrial mouse model

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Studies on mitochondrial DNA (mtDNA) mutational heteroplasmy in oocytes or embryos are limited due to the low abundance of mtDNA. Preimplantation genetic diagnosis (PGD) has been suggested as a way to determine mtDNA heteroplasmy in oocytes and embryos to avoid transmission of heritable mtDNA diseases. We aimed to determine the level of mtDNA heteroplasmy in single blastomeres, TE and blastocysts for PGD in heteroplasmic mitochondrial mouse model.

**STUDY DESIGN, SIZE AND DURATION:** We explored the suitability of PGD by comparing the level of mtDNA heteroplasmy between first PBs and MII oocytes (n = 33), between first PBs, second PBs and zygotes (n = 30), and between first PBs, second PBs and their corresponding blastomeres of 2- (n = 10), 4- (n = 10) and 8-cell embryos (n = 11). Levels of mtDNA heteroplasmy in second PBs (n = 20), single blastomeres from 8-cell embryos (n = 20), TE (n = 20) and blastocysts (n = 20) were also compared.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** Heteroplasmic mice (BALB/cByJ and NZB/OlaHsd) were used in this study. The first PBs were biopsied from in vivo matured MII oocytes. The ooplasm was then subjected to ICSI. After fertilization, second PBs were biopsied and zygotes were cultured to recover individual blastomeres from 2-, 4- and 8-cell embryos. Similarly, second PBs were biopsied from in vivo fertilized zygotes and single blastomeres were biopsied from 8-cell stage embryos. The remaining embryo was cultured until the blastocyst stage to isolate TE cells. Polymerase chain reaction followed by restriction fragment length polymorphism was performed to measure the level of mtDNA heteroplasmy in individual samples.

**MAIN RESULTS AND THE ROLE OF CHANCE:** Modest correlations and wide prediction interval [PI at 95% confidence interval (CI)] were observed in the level of mtDNA heteroplasmy between first PBs and MII oocytes (r² = 0.56; PI = 45.96%) and zygotes (r² = 0.69; PI = 37.07%). The modest correlations and wide PI were observed between second PBs and their corresponding blastomeres of 2- (r² = 0.42; PI = 48.04%), 4- (r² = 0.42; PI = 48.04%) and 8-cell embryos (r² = 0.42; PI = 48.04%). A strong correlation with a narrow PI was observed among individual blastomeres of 2-, 4- and 8-cell stage embryos (r² = 0.92; PI = 11.73%, r² = 0.92; PI = 11.73%, r² = 0.92; PI = 11.73%).
Moreover, single blastomeres from 8-cell stage embryos showed a close correlation and an intermediate PI with corresponding TE cells ($r^2 = 0.81; \text{PI} = 28.15\%$) and blastocysts ($r^2 = 0.76; \text{PI} = 36.43\%$).

**LIMITATIONS, REASONS FOR CAUTION:** These results in a heteroplasmic mitochondrial mouse model should be further verified in patients with mtDNA disorders to explore the reliability of PGD.

**WIDER IMPLICATIONS OF THE FINDINGS:** To avoid the transmission of heritable mtDNA disorders, PGD techniques should accurately determine the level of heteroplasmy in biopsied cells faithfully representing the heteroplasmic load in oocytes and preimplantation embryos. Unlike previous PGD studies in mice, our results accord with PGD results for mitochondrial disorders in humans, and question the reliability of PGD using different stages of embryonic development.

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**Keywords:** mitochondrial DNA / mtDNA disorders / mtDNA heteroplasmy / preimplantation embryos / preimplantation genetic diagnosis

**Introduction**

Mitochondria are cellular organelles responsible for the production of energy in the form of adenosine triphosphate by a process called oxidative phosphorylation (OXPHOS) (Hudson and Vinograd, 1967; Wallace, 1999). Defects in the OXPHOS system lead to mitochondrial diseases affecting single tissues or multiple systems. Mammalian mitochondrial DNA (mtDNA) is small (~16.6 kb in humans), circular and double-stranded extra-chromosomal DNA located in the mitochondria (Nass, 1966) and is exclusively maternally inherited (Birky, 2001). Since post-fertilization autophagy of sperm organelles prevents the transmission of paternal mtDNA (Al Rawi et al., 2011), mammalian individuals maintain a single mtDNA genotype of maternal origin, known as ‘homo-plasmy’. However, it may occur that >1 mtDNA genotype is found within the same cell, a condition termed as ‘heteroplasmy’.

Most of the pathogenic mtDNA mutation disorders are heteroplasmic in nature, with cells harbouring both wild-type and mutated mtDNA. Nearly, 1 in 5000 individuals is affected by a pathogenic mtDNA mutation (Skladal et al., 2003; Poulton et al., 2010). For phenotypic expression of the disorder, a critical threshold of heteroplasmcy must be crossed (Howell, 1983; Chinnery et al., 1997; Jeppesen et al., 2006), which is not only dependent on the specific mutation but also on the tissue (Monnot et al., 2011). Nevertheless, it is difficult to predict the mutation load based on the clinical manifestation of a disease, and this also holds true in the opposite direction.

Since the level of mtDNA heteroplasma may vary dramatically between a mother and her offspring, asymptomatic mothers with low-to-medium levels of heteroplasmy can give birth to diseased children with higher heteroplasmic loads (Holt et al., 1989; Larsson et al., 1992; Blok et al., 1997). mtDNA heteroplasma varies between and within tissues, both in germ-line and in somatic cells (Jenuth et al., 1997; Steffann et al., 2007; Monnot et al., 2011, 2013) and this heterogeneous distribution may cause variation in the severity of phenotypes in many disorders. It is believed that random or biased segregation of heteroplasmic mtDNA genotypes during embryogenesis leads to the large variability in the level of heteroplasma between oocytes and embryos within an individual (Blok et al., 1997; Brown et al., 2001; Steffann et al., 2007).

Preimplantation genetic diagnosis (PGD) aims to select mutation-free oocytes or embryos by analysing the level of heteroplasma in biopsied polar bodies (PBs), blastomeres or trophoectoderm (TE) cells (Hellebrekers et al., 2012). In cases where mutation-free specimens are absent, oocytes or embryos with the lowest mutant load are selected for transfer, thus minimizing the risk of inheriting the disease (Treff et al., 2012). An obvious prerequisite for applying PGD is that the heteroplasmic load determined in the analysed cells is a faithful indicator of the heteroplasmic load in the embryo proper. Analysis of first PBs and their corresponding ooplasm of mature metaphase II (MII) oocytes in heteroplasmic mice showed a strong correlation in the level of mtDNA heteroplasma (Dean et al., 2003). Similarly, analysis of second PBs and their corresponding zygotes in trans-mitochondrial mice also showed a strong correlation (Sato et al., 2005). However, PGD in human mitochondrial encephalopathy, lactic acidosis and stroke-like episodes patients has shown poor correlation in the mutant load between first PBs and corresponding oocytes and embryos (Gigarel et al., 2011; Vandewoestyne et al., 2012), thus questioning the reliability of PGD.

The present study aims to investigate the reliability of PGD in a BALB/OlaHsd mixed strain mouse model characterized by the co-occurrence of two polymorphic mtDNA sequence variants coming from NZB/OlaHsd and BALB/cByJ mice (Jenuth et al., 1996). Previous studies were limited to a comparison between first PBs and oocytes and embryos or between second PBs and zygotes. To our knowledge, this report is the first to compare first and second PBs against their corresponding oocytes, zygotes and blastomeres from different cleavage-stage embryos, to assess the variation among individual blastomeres of different cleavage stages and to compare second PBs, single blastomeres of 8-cell embryos, TE and blastocysts.

**Materials and Methods**

**Animal models and ethical approval**

Heteroplasmic BALB/OlaHsd females (kindly provided by Dr B.J. Battersby, FinnMIT Academy of Finland Centre of Excellence, University of Helsinki) were crossed with homoplasmic BALB/cByJ male mice (Charles River Laboratories, Brussels, Belgium). All animal procedures and experiments were approved by the Animal Ethics Committee of the Ghent University Hospital (Project ECD No. 11/21), Ghent, Belgium.
Oocytes and zygotes recovery

Heteroplasmic female mice, 7–14 of weeks age, were stimulated with 7.5 IU pregnant mare’s serum gonadotrophin (PMSG, Folligon, Intervet, Boxmeer, The Netherlands), followed by 7.5 IU hCG (Chorulon, Intervet, Boxmeer, The Netherlands) 46–48 h later. In vivo matured MII oocytes were harvested 12–14 h after hCG injection, whereas in vivo-produced zygotes were recovered 19–21 h after hCG injection in HEPES-buffered potassium simplex optimized medium (hereafter called KSOM–HEPES, containing 0.2 mM glucose and supplemented with 4 mg/ml bovine serum albumin (BSA, Calbiochem). Cumulus cells surrounding the oocytes were removed by treatment with 200 IU/ml hyaluronidase in KSOM–HEPES. All oocytes and zygotes were cultured under paraffin oil at 37°C in 6% CO2 and 5% O2 in KSOM medium containing 5.5 mM glucose and 4 mg/ml BSA (hereafter called standard culture conditions).

Micromanipulation

Micromanipulation was performed in 5 µl droplets of KSOM–HEPES in plastic petri dishes (model-35002; Falcon Becton Dickinson Labware, Franklin Lakes, NJ, USA) mounted on an inverted microscope (Olympus IX-70; NY/NJ Scientific, Inc., USA) equipped with two electric/hydraulic micromanipulators (model: MM-188 and MO-109; Narishige, NY/NJ Scientific, Inc.). A heated stage was used for partial zona dissection (PZD), PB biopsy, blastomere biopsy and TE biopsy, while ICSI was done on a cooled stage.

PZD was performed by penetrating the zona pelludica (ZP) using a sharp glass micro-needle creating a tangential slit in the ZP. A biopsy pipette (15–20 µm inner diameters) was inserted into the perivitieline space through this slit, and each PB was gently aspirated into the biopsy pipette. Immobilized frozen–thawed spermatozoa were injected into the ooplasm using a piezo-driven ICSI set-up in KSOM–HEPES supplemented with 20% fetal bovine serum (Gibco BRL, Carlsbad, CA, USA). Five to six hours after ICSI, the fertilized oocytes were subjected to the second PB biopsy. The zygotes were cultured in KSOM at 37°C in 5% O2 and 6% CO2 for subsequent embryonic development. Individual blastomeres from 2-, 4- and 8-cell embryos were recovered 10–14, 32–36 and 44–48 h, respectively, following second PB biopsy. The ZP was removed from embryos by a short incubation in acid Tyrode’s (AT) solution and subsequent washing in KSOM–HEPES. Each zona-free embryo was incubated until the blastocyst stage. Protruding trophectoderm (TE) cells (5–10) from hatching blastocysts were biopsied by laser-assisted micromanipulation. Heteroplasmic loads in second PBs, single blastomere, TE biopsy and blastocyst were assessed.

Sample collection

Heteroplasmic load was compared between (i) first PBs and the ooplasm of mature MII oocytes, (ii) first PBs, second PBs and zygotes, (iii) first PBs, second PBs and blastomeres of (a) 2-, (b) 4- and (c) 8-cell embryos, and between (iv) second PBs, single blastomeres from 8-cell embryos, TE and blastocysts (Fig. 1). In the first set of experiments, the first PB and ooplasm of a mature MII oocyte were isolated by exposing MII oocytes to AT solution for 15–30 s at room temperature. In the second set of experiments, first PBs were biopsied by micromanipulation before subjecting the MII oocytes to ICSI. Five to six hours after ICSI, the second PBs were isolated from zygotes by exposing them to AT solution.

In a third series of experiments, first and second PBs were biopsied and the zygotes were cultured for subsequent blastocyst recovery. For blastomere harvesting, embryos were briefly treated with AT solution for removal of the ZP and then washed in KSOM–HEPES. Each zona-free embryo was incubated in Ca2+–Mg2+-free medium and individual blastomeres from 2-, 4- and 8-cell embryos were aspirated by a glass pipette. In a final experimental series, second PBs were biopsied from in vivo-produced zygotes, which were then further cultured. A single blastomere was biopsied at the 8-cell stage and the embryos were further cultured until the blastocyst stage, from which a small pool of TE cells was isolated by laser-assisted micromanipulation.
**DNA extraction**

Collected specimens (PBs, oocytes, zygotes, blastomeres, pool of TE cells and blastocysts) were individually transferred into 200 μl micro-centrifuge tubes (Westburg, Leusden, The Netherlands) containing 10 μl of PicoPure DNA extraction buffer with proteinase K (PicoPure DNA extraction kit, Artcurus, Mountain View, CA, USA). All samples were incubated at 65°C for 3 h, centrifuged briefly and heated at 95°C for 10 min to inactivate proteinase K.

**Polymerase chain reaction**

Heteroplasmic BALB/OlaHsd mice harbour a mixture of mtDNA derived from NZB/OlaHsd and BALB/cByJ strains. PCR amplification was performed using the 6-fluorescent amidite (6-FAM) labelled forward primer MT9 (5'-GAGCATCTTATCCAGCCTCC-3'), annealing the mitochondrial genome from nucleotide (nt) 3571 to 3591, and the backward primer MT10 (5'-CTGCTTCAAGGTATCGTGGGT-3') (labelled with (1-Naphthyl)ethylenediamine dihydrochloride (NED), but not used for detection in the current study), annealing the mitochondrial genome from nt 4059 to 4079. PCR was performed in a reaction volume of 20 μl containing 10 μl of mtDNA extract and 10 μl of the reaction mixture. The reaction mixture consisted of 1.0 μl forward primer, 1.0 μl backward primer, 10 mM deoxynucleotide triphosphate mix (Applied Biosystems), 200 μM of 10× PCR buffer (Qiagen, Germantown, MD, USA), 0.3 μg/μl of BSA (Sigma Aldrich, Bornem, Belgium), 0.5 mM of MgCl2 (Qiagen) and 1.3 U of Hot Start Taq DNA polymerase (Qiagen). Conditions used to amplify the target sequence were as follows: a 5 min hot start at 94°C, followed by cycling at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final elongation at 72°C for 5 min. Oocytes and zygotes were run for 32 PCR cycles, whereas TE cells, blastocysts, PBs and blastomeres from 2-, 4- and 8-cell stage embryos were run for 44, 40, 34, 36 and 38 PCR cycles, respectively.

**Restriction fragment length polymorphism**

Ten microlitres of Rsal restriction enzyme mix was added to 10 μl PCR amplified product. The restriction digest mixture was composed of 0.5 μl Rsal (10 U/μl, Promega Corporation, Madison, WI, USA), 0.2 μl BSA (10 μg/ml, Promega), 2 μl restriction enzyme 10× buffer C (Promega) and 7.3 μl sterile, deionized water. Digestion was achieved after incubating PCR amplified products at 37°C for 4 h. Samples were stored at −20°C until further analysis. The amplified mtDNA fragments were analysed by capillary electrophoresis using an ABI 3100 Genetic Analyzer (Applied Biosystems).

**Heteroplastic load measurement**

Heteroplastic amplicons were digested into a 6-FAM labelled 121-bp fragment (exclusively coming from BALB mtDNA), an NED-labelled 170-bp fragment (common to NZB and BALB mtDNA) and a 6-FAM-labelled 339-bp fragment (exclusively coming from NZB mtDNA) (Supplementary data, Fig. S1). The NZB heteroplastic load (%) was determined by calculating the ratio of the peak area of the 6-FAM 339-bp fragment and the sum of the peak areas of the 6-FAM 339-bp fragment and the 6-FAM 121-bp fragment, multiplied by 100.

**Statistical analysis**

Statistical analyses were performed by using Statistical Package for the Social Sciences (SPSS Statistics 21, IBM Corp., NY, USA). Ninety-five percentage of prediction intervals (PIs) and coefficient of determination ($r^2$) were measured by linear regression analysis. Microsoft Excel was used to calculate mean, standard deviation and coefficient of variation (CV).

**Results**

**Validation of the technique**

The PCR–RFLP technique for measuring the levels of mtDNA heteroplasmy was validated in artificially composed heteroplastic samples prepared by mixing an equal concentration of BALB and NZB mtDNA. Mixtures (25, 50 and 75%) were prepared by combining 25, 50 and 75 μl of NZB mtDNA with 75, 50 and 25 μl of BALB mtDNA, respectively. Different dilution series of 1 : 10, 1 : 20 and 1 : 50 were also prepared. All measurements were performed in triplicate samples. Positive (DNA from BALB/cByJ mouse tails) and negative controls (without template mtDNA) were used to detect contamination. A strong correlation ($r^2 = 0.98$) was seen between the input and output level of mtDNA (NZB/S) heteroplasmy.

A detailed summary of all the following results is shown in Table I.

**Comparison between PBs and corresponding oocytes and zygotes**

In a first set of experiments, the level of mtDNA heteroplasmy was compared between 33 oocytes and their corresponding first PBs, and between 30 zygotes and corresponding first and second PBs. A wide PI (PI = 45.96%) and a high CV (CVav = 11.8 ± 6.9%) were found between first PBs and corresponding oocytes (Supplementary data, Fig. S2). In two-thirds of these cases (67%), the level of heteroplasmy in first PBs and corresponding oocytes differed by <10%. In the other instances, the difference in the heteroplastic load between these two samples ranged from 11 to 27%. We observed a similar PI and CV between zygotes and corresponding first (PI = 37.07% and CVav = 8.1 ± 7.0%) and second PBs (PI = 39.70% and CVav = 7.8 ± 6.1%) (Supplementary data, Fig. S2). In 73% of the measurements, the heteroplastic load between both PBs and zygotes varied by <10%.

**Comparison between PBs and blastomeres**

Heteroplastic loads in all individual blastomeres of 2- ($n = 10$), 4- ($n = 10$) and 8-cell ($n = 11$) embryos were compared with their corresponding first and second PBs. A wider PI between first PBs and corresponding blastomeres of 2- (PI = 22.37% and CVav = 5.5 ± 4.1%), 4- (PI = 24.72% and CVav = 8.7 ± 6.1%) and 8-cell embryos (PI = 46.04% and CVav = 7.7 ± 4.5%) was found (Supplementary data, Figs S2 and S3) compared with second PBs and their counterparts (PI = 17.18% and CVav = 7.4 ± 7.2%, PI = 24.39% and CVav = 8.7 ± 6.1%, PI = 34.80% and CVav = 8.1 ± 3.0%, respectively) (Supplementary data, Figs S2 and S3). Interestingly, an intermediate PI was observed in the level of mtDNA heteroplasmy between first PBs and their corresponding second PBs (PI = 22.43%) (Supplementary data, Fig. S3). The average CV between them was 6.7 ± 7.8%. In most of the cases (89%), first PBs and second PBs varied by <10%.

**Inter-blastomere variation**

The width of the PI was less among individual blastomeres of 2-, 4- and 8-cell embryos compared with that between PBs and their counterparts. However, the PI continuously increased with increasing cleavage stage: 2-cell embryos: PI = 11.73%, 4-cell embryos: PI = 18.85% and 8-cell embryos: PI = 21.42% (Fig. 2). The average CV between blastomeres

 resultados
Table I  Summarized results showing the comparison of mtDNA heteroplasmy between all experimental groups.

<table>
<thead>
<tr>
<th>Comparison between</th>
<th>Width of prediction interval (PI) (at 95% CI)</th>
<th>Coefficient of determination</th>
<th>Difference in the level of mtDNA heteroplasmy (%)</th>
<th>CV (%)</th>
<th>References</th>
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<tr>
<td></td>
<td>Maximum</td>
<td>Minimum</td>
<td>Mean</td>
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<td>Cell type 2</td>
<td></td>
<td></td>
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<td>First PBs</td>
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<td>26.68</td>
<td>23.08</td>
<td>23.58</td>
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</table>

Strong correlations were observed between individual blastomeres of 2-, 4- and 8-cell embryos and also between trophectoderm and blastocysts, with a narrow width of PI. Variations in the range of mtDNA heteroplasmy and CV were also narrow between them compared with other groups.

ICM, inner cell mass.
was 4.2 ± 3.1% in 2-cell embryos, 7.1 ± 5.9% in 4-cell embryos and 5.6 ± 2.8% in 8-cell embryos. Inter-blastomere variation was < 10% in all (10/10), in 90% (9/10) and in 60% (6/10) of the cases for 2- (0.2–6.1%), 4- (0–12.1%) and 8-cell embryos (0–17.3%), respectively.

**Comparison between second PBs, blastomeres, TE and blastocysts**

Second PBs (n = 20), single blastomeres of 8-cell embryos (n = 20), TE (n = 20) and bulk remaining of whole blastocysts (n = 20) coming from the same embryos were analysed. Wide PIs and high CVs were seen between second PBs and their corresponding single blastomeres of 8-cell embryos (PI = 48.04%), TE cells (PI = 54.79%) and blastocysts (PI = 57.48%) (Supplementary data, Fig. S3), with an average CV of 11.6 ± 7.6, 13.6 ± 10.5 and 13.0 ± 10.5%, respectively. However, intermediate PIs were seen between single blastomeres of 8-cell embryos and their corresponding TE cells and blastocysts (28.15 and 36.43%), respectively (Supplementary data, Fig. S3), with an average CV of 5.9 ± 5.7% and 6.9 ± 7.7% between them. In 45% (9/20) of the cases, the heteroplasmic load varied by >10% between second PBs and corresponding blastomeres, TE cells and blastocysts. However, in 90% of the cases (18 of 20), the variation in the heteroplasmic load was <10% between blastomeres and their corresponding TE cells. Similarly, in 75% of the instances (15 of 20), the heteroplasmic load varied by <10% between single blastomeres at 8-cell stage and their corresponding blastocysts. Furthermore, a narrow PI was found between TE and blastocysts (23.58%) (Fig. 2), with an average CV of 5.3 ± 5.5%. In 90% of these cases (18 of 20), the heteroplasmic load varied by <10%.

**Discussion**

A modest correlation and a wider variability in the level of mtDNA heteroplasmy were seen in this study between first PBs and their corresponding oocytes, which is in contrast to the strong correlation found by Dean et al. (2003) in heteroplasmic mice. Moreover, we have found an increased variation in the range of heteroplasmic load between the biopsied first PBs and oocytes (0.1–26.7%), compared with their results...
In contrast to Dean et al., in this study, we incorporated fluorescently labelled primers in combination with capillary electrophoresis for a more accurate readout. Our findings support those of Gigarel et al. (2011) who showed poor correlations in the levels of mutant mtDNA in heteroplasmic first PBs and their corresponding oocytes and embryos in human samples. In only half of their cases, first PBs and their counterparts had similar mutant loads (± 10%). They also showed that in the high-end range of PB mutations, the mutation load was generally higher in the PB than in its corresponding oocyte, in contrast to the present study, where the heteroplasmic load distribution was completely random.

In contrast to Sato et al. (2005) a poor correlation and a wider variability were found between zygotes and their corresponding second PBs in this study. In transmitochondrial mice, these authors found a higher least-squares correlation coefficient for the proportions of mtDNA in second PBs and zygotes (Table I). The difference could be due to the use of different mouse models. They used mito-mice carrying a large-scale deletion, whereas we have used heteroplasmic BALB mice with a neutral polymorphism.

Interestingly, we observed an increasing trend in the segregation of heteroplasmic load in individual blastomeres from 2-, 4- and 8-cell stage embryos, as was also seen in non-human primates by Lee et al. (2012) (Table I). However, they observed a much wider inter-blastomere variation in contrast to our results. The occasional variability seen in the later developmental stages resembles that seen also in humans (Monnot et al., 2011; Vandewoestyne et al., 2012; Sallevelt et al., 2013). This escalating variation is likely due to the random drift of mtDNA molecules during successive cell divisions. Such increased heteroplasmic mtDNA dispersion with iniating blastomere counts makes the PGD technique on single-blastomere biopsies less reliable, as reported earlier (Vandewoestyne et al., 2012; Sallevelt et al., 2013).

A major difference with previous studies in mice is that we incorporated fluorescently labelled primers in combination with capillary electrophoresis. One hypothesis for the discrepancy in results could be that mitochondria are unevenly distributed in an oocyte or in a zygote, thus leading to varying mitochondrial complements in different blastomeres (Van Blerkom and Runner, 1984; Van Blerkom et al., 2000). Another hypothesis could be that random genetic drift predominantly determines the transmission of heteroplasmic mtDNA irrespective of developmental stages and individual subjects (Brown et al., 2001; Monnot et al., 2011). Hence, this study shows that the two types of mtDNA in a polymorphic animal do not segregate evenly during embryogenesis.

The present study shows a close correlation in the level of heteroplasmy between biopsied TE cells and their corresponding blastocysts. Treff et al. (2012) found a strong correlation between biopsied TE cells and corresponding ICM cells in humans, indicating a faithful representation by the TE for the embryo proper (Table I). However, Lee et al. (2012) found a wider variation in levels of heteroplasmy between TE cells and ICM in non-human primates.

Unlike a few studies in mice which reported a strong correlation between PBs and their counterparts, our results coincide with the rather modest correlation seen in humans with mtDNA mutation disorders (Gigarel et al., 2011; Vandewoestyne et al., 2012) making PBs not readily suitable for PGD. In spite of the strong correlation among individual blastomeres, occasional inter-blastomere variability makes PGD based on single-blastomere biopsy risky. Given that variability increases with progressive cleavage stage of preimplantation embryos, analysing multiple cells, e.g. multiple blastomeres or multiple TE cells, would be expected to be more accurate. However, biopsy of multiple blastomeres on Day 3 would be very invasive and detrimental for subsequent embryonic developmental potential (De Vos et al., 2009) compared with TE biopsy on Day 5. In this study, levels of heteroplasmy in TE were shown to correlate strongly with that of the whole blastocyst. Therefore, PGD based on TE biopsy seems more reliable and safer for estimating the level of mtDNA heteroplasmy in a clinical context. Taking into account the single cell being analysed in earlier stages compared with multiple cells, which can be taken from an advanced stage of preimplantation development, analysing a pool of TE cells addresses the issue of probable heterogeneity among the TE cells. However, further studies on human patients should be performed to optimize the procedure and to ensure the safety and reliability of PGD in order to prevent the transmission of heritable mtDNA disorders.

**Supplementary data**

Supplementary data are available at http://humrep.oxfordjournals.org/.

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**Authors’ roles**

J.N. designed and performed the experiments, collected and analysed data and wrote the manuscript. B.H., M.V., R.C., J.G. and T.D. designed, conceived and supervised the experiments and revised the manuscript. S.G. and Y.L. performed the experiments, and collected and analysed the data. C.Q. and S.L. performed the experiments. D.D. and P.D.S. conceived, designed and supervised the experiments and revised the manuscript.

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**Conflict of interest**

None declared.

**References**


